Neurobehavioral toxicity produced by sodium fluoride in drinking water of laboratory rats

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Abstract: The effect of exposure to different concentrations of sodium fluoride (Na-F) for different durations on learning and memory tasks in rats (non-associative and associative learning) was assessed in our study. Three groups of fifteen pregnant Wistar female rats each, were administered Na-F in drinking water at one of three concentrations; 0, 50 and 100 ppm from second trimester of pregnancy till weaning of their pups at 30 days of age. Pups were then allocated into 5 groups of 20 animals each, where Na-F was administered in three different concentrations with different exposure periods throughout the study. Brain tissue specimens, representing all treatment groups, were taken for histopathological examination. The average body weight gain was significantly lower in group of rats exposed to high Na-F doses for long duration, with distinct hair loss. Open field revealed a significant influence of dose of Na-F on exploratory motor activities (EMA) and emotionality with marked impairment in habituation in rats exposed to high Na-F. Moreover, learning and memory assessed during maze test showed reduced memory retention in rats exposed to high Na-F for long periods. In novelty acquisition test, despite evidence of occurrence of habituation in all groups, a noticeable reduced degree was demonstrated in rats continued to administer high Na-F for long duration. Furthermore, histopathological evaluation revealed distinct neurodegenerative changes of nerve cells especially in hippocampus. Our results suggest that exposure of rats to Na-F in high doses for long duration has detrimental effects on the brain as reflected in diminished learning and memory. [Journal of American Science 2010;6(5):54-63]. (ISSN: 1545-1003).

Keywords: Neurobehavioral-toxicity-sodium fluoride-drinking water - laboratory rats

1. Introduction

Chronic fluoride toxicity represents a hazard to human health. The fluoride administered during gestation can cross both human and rat placenta and is also present in mother's milk (Drinkard et al., 1985; Fassman, 1993; Hassunuma, 2007). Excessive exposure to fluoride has been reported to be associated with central nervous system dysfunction manifested in lethargy, insomnia and deterioration of learning and memory (Spittle, 1994, 2000, Wu et al., 2006; Sharma et al., 2009). Moreover, chronic fluorosis reduced mental work capacity of adults as well as Intelligence Quotient (IQ) of children (Zhao et al., 1996; Lu et al., 2000; Xiang et al., 2003). Comparable effects are also reported with animal studies in rats (Niu et al., 2008; Gao et al., 2009).

Moreover, chronic exposure to high concentrations of sodium fluoride has been reported to induce disturbances in the development of brain in offspring rats (Liu et al., 1989). Rats exposed to flurosis showed a number of histopathological changes in the brain, including demyelinization, a decrease in the number of Purkinje cells, thickening and disappearance of dendrites, swelling of mitochondria, and dilation of endoplasmic reticulum in neurons (Guan et al., 1986, 1998).

Non-associative behavioural habituation affords one of the most essential forms of learning, both in animals and in humans. Open field is a potentially useful model for simultaneous assessment of anxiety and memory (Weiss and Greenberg, 1996; Weisstaub et al., 2006). Rats submitted for the first time to an open field display higher spatial exploration, a form of information storage (Eichenbaum, 1996), than in successive exposures. Thus, the decrement in response to successive exposures is taken as an index of memory of habituation (Izquierdo et al., 2001; Winograd and Viola, 2004).

Exposure to a novel environment also generates novelty recognition process that involves increased awareness with matching the stored memories of formerly explored places with the new spatial information to judge its novelty (Montag-Sallaz et al., 1999).

Formation of association between choice alternatives and their dependant outcomes is an important aspect of learning that may be sensitive to hippocamal dysfunction (Mahut et al., 1982; Reilly and Good, 1989; Johnson et al., 2008). Many studies in mammals have confirmed the involvement of hippocampus in some forms of associative and non-associative memories (Izquierdo and Medina, 1997;

Zhu et al., 1997; Thiel et al., 1998; Eichenbaum, 1999; McGaugh, 2000).

A deteriorating effect has been reported for sodium fluoride on performance in some memory tasks such as open field habituation in rats (Pereira and Dombrowski, 2009).

To our knowledge, no literatures are available to address the effect of Na-F, regarding the association between different doses and exposure periods, on learning and memory in rats. Furthermore, the involvement of various arrays of measurements to properly evaluate both associative and non-associative learning abilities in rats is not well implemented.

Thus, the objectives of the current study were to investigate the influence of exposure to different concentrations of sodium fluoride during gestation and postnatal period in rats on learning and memory tasks. Moreover, since the hippocampus is greatly involved in the process of learning and memory, histopathological examination was also carried out in order to detect alterations in brain tissues.

2. Material and Methods

2.1. Animals and housing

Forty five mature Wistar female rats weighing about 200-220g were obtained from the Unit for Laboratory Animals at Faculty of Veterinary Medicine, Cairo University and used in our study. They were housed in polypropylene cages with stainless steel wire lids (bedded with wood shavings) and maintained on a standard laboratory feed diet throughout the course of the study. Animals had free access to feed and water and housed at a room temperature of 20-22°c, 60% humidity on a 12h light:dark cycle. All females were mated with males of the same strain. Animal care was in compliance with applicable guidelines from Cairo University Policy on Animal Care and Use.

2.2. Administration of sodium fluoride:

Pregnant females were divided at random into three groups of 15 animals each and received Na-F at one of three different concentrations; 0 (control), 50 and 100 ppm on a mg/kg/day basis of 0, 5.15 and 10.77 Na-F, respectively). Sodium fluoride (Na-F, Sigma Chemical Company) was incorporated in drinking distilled water and administered to pregnant rats for a 44 days period (from day 8 of gestation till termination of lactation and weaning of pups at 30 days of age). After weaning, all pups were then collected and distributed into five groups of 20 animals each, divided on 2 replicates, as following: Group (1) control, n=20: weanling pups were derived from control dams receiving no Na-F. These pups served as a control group, where Na-F-free water was administered throughout the study till completing all assessments of learning and memory behaviours at 105 days of age.

Group (2) low-discontinued (LD), n=20: weanling pups were derived from dams receiving low dose of Na-F. Pups were then exposed to *ad libitum* supply of low dose of Na-F in drinking water, only till weaning at 30 days of age. Group (3) low-continued (LC), n=20: weanling pups were derived from dams receiving low dose of Na-F. Pups were then continually exposed to *ad libitum* supply of low dose of Na-F in drinking water till completing all assessments of learning and memory behaviours at 105 days of age. Group (4) high-discontinued (HD), n=20: weanling pups were derived from dams receiving high dose of Na-F. Pups were then exposed to *ad libitum* supply of high dose of Na-F in drinking water, only till weaning at 30 days of age. Group (5) high-continued (HC), n=20: weanling pups were derived from mothers receiving high dose of Na-F. Pups were then continually exposed to *ad libitum* supply of high dose of Na-F in drinking water till completing all assessments of learning and memory behaviours at 105 days of age.

2.3. Body weight gain

All pups were weighed at the onset of treatment, on day 30, and then individual body weight of all rats per group was recorded weekly throughout the study and body weight gain was calculated as the difference between final and initial weight. Mortalities were recorded as it occurred.

2.4. Behavioural testing

Behavioural testing started at 60 days and ended at 105 days of animals age.

2.4.1. Open field habituation test

The locomotor activity and habituation, a form of non-associative learning, were measured in the open field test (Kelly, 1993; Mello e Souza et al., 2000; Chioca et al., 2008). The open field used was a square wooden arena measured (90 x 90 x 25cm. The wood of the apparatus is covered with a plastic laminate (formica), which prevents absorption of fluids (urine of rats). The floor was divided by black lines into 36 small squares (15 x 15cm). All testing was conducted between 09:00 and 15:00 h. All treatments groups were tested at the same day in a random order. Rats were gently placed into a corner of the arena and allowed to explore the apparatus for 3 minutes. During the three minutes of exploration, the time spent freezing (no movement) was quantified. Exploratory measures as well as non-exploratory behaviours were recorded by the observer (Kalueff et al., 2006). Exploratory motor activity (EMA) measures included horizontal locomotion (the number of squares crossed) as well as vertical activity (rearing). A crossed square was defined as the rat placing its two forepaws in the next square and moving forward (Chioca et al., 2008), while

vertical activity was defined as the number of times an animal stood erect on its hind legs with its fore legs in the air or leaning against the wall of the open field (Brown et al., 1999). Non-exploratory measurements comprised only the vegetative behaviours (numbers of urination episodes and defecation boli). After the 3 minutes test session, the rat was returned to its home cage and the open field was cleaned using 70% ethyl alcohol (to avoid odour cues) and permitted to dry between tests. To assess the process of habituation to the novelty of arena, rats were exposed to the apparatus for a 3 minutes test session, on three consecutive days.

2.4.2. Classic maze test

Associative learning was assessed using classic maze test. The base of the maze measured (100 x 100cm) with walls height of 25cm. The entire maze was made of plywood with a glass cover in order to prevent escape of animals and allow observation. Testing was carried out between 09:00 and 15:00 h, where all groups were randomly allowed for testing at the same day. Rats were deprived from feed for a 23 hours period before start of testing. Rats were given their daily feed amount as a reward at the end of the maze. Animals were given one trial per day for five consecutive days. Time elapsed to locate the feed at the end and numbers of entries of blind alleys were recorded according to Staddon (1983).

2.4.3. "Novelty acquisition for exploration" test

We designed to investigate the exploratory activity, where a "mini-holeboard" consisted of a dark platform (40 x 40cm) containing a hole (5.5cm diameter x 5cm depth) in each quadrant was used. This mini-holeboard was inserted into the base of the recording chamber (40 x 40 x 40cm). A small object, which differed in scent and texture was placed in each hole (stimulus rich). Exploratory behaviour of rats including numbers of rears as well as head dips (to examine the interior of, or the objects within the four holeboard holes) were counted during a 15-min exposure period of the rats to the holeboard. To assess whether learning has occurred in our rats, re-exposure of rats to the same environment was conducted, 14 days later and the same parameters were recorded (Manahan-Vaughan and Braunewell, 1999).

2.5. Histopathological examination

After completing all behavioural assessments, tissue specimens were taken from the brain for histopathological examination. The specimens were fixed in 10% neutral buffer formalin, processed by paraffin embedding method, sectioned at 4-5 um and stained with Hematoxylin and Eosin stain (Bancroft et al., 1996). Axons were examined by using Bielschowsky's stain (Louis and Williams, 1995).

For Immunohistochemical examination, Paraffin sections of brain tissues were immunostained according to Ultra vision detection system (Anti-Polyvalent, HRP/ DAB), using monoclonal antibodies of Glial fibillary Acidic Protein (GFAP Ab-1, NeoMarkers, Fremont, CA) for determining astrocytes (Overmyer et al., 1999).

2.6. Statistical analysis

Data for all variables were subjected to analyses of variance (ANOVA) to assess the effect of dose of Na-F administered to rats, duration of administration as well as session factor for behavioural tests using the general linear models procedure in SPSS® statistical software (SPSS, 2006). When ANOVA was significant, post hoc Tukey HSD test was conducted for individual comparisons. A probability of $p<0.05$ was considered significant for all evaluations. All data are expressed as mean ± SEM.

3. Results

3.1. Body weight gain

The averages of rats' body weight gain allover the study was shown in (Fig. 1). Both dose of Na-F administered to rats ($F_{(1, 45)} = 4.86$; $p = 0.03$) and duration of exposure ($F_{(1, 45)} = 6.04$; $p = 0.02$) had a significant effect on average body weight gain of rats. Lower body weight gain was significantly seen in groups of animals exposed to high concentrations of Na-F for long period (59.91±4.33 g) compared to those exposed to low doses of Na-F for short period (81.76±5.74 g) and rats in control group (85.59±5.45 g).

3.2. Open field test

Only dose of Na-F administered to animals, but not duration of exposure, had a significant influence on exploratory motor activity assessed on first occurrence in the open field test. Although no significant dose difference was found for horizontal activity (numbers of crossed squares; $F_{(1, 45)} = 2.45$; $p = 0.13$), a significant dose effect was noted for the vertical one (numbers of rearing; $F_{(1, 45)} = 7.88$; $p = 0.01$). Correspondingly, regardless of Na-F exposure time, rats exposed to different Na-F concentrations demonstrated a significant different profile of novelty-induced anxiety related behaviours, as measured by time spent freezing and vegetative behaviours. There was a marginally significant dose effect in duration of freezing, with rats administered high Na-F doses spent more time freezing than their counterparts in control group ($F_{(1, 45)} = 3.82$; $p = 0.05$). For vegetative behaviours, rats received high Na-F doses defecated more frequently than rats of other treatments ($F_{(1, 45)} = 10.32$; $p = 0.002$), while no differences were recorded

for numbers of urination episodes ($F_{(1, 45)} = 1.33$; $p = 0.25$).

Habituation over the course of three successive test sessions of open field revealed a significant session factor diminishing effect on the following parameters; numbers of crossed squares ($F_{(2, 135)} = 7.65$; $p = 0.00$), numbers of rearing ($F_{(2, 135)} = 11.54$; $p = 0.00$), time spent freezing ($F_{(2, 135)} = 8.29$; $p = 0.00$), and defecation boli ($F_{(2, 135)} = 7.92$; $p = 0.00$) but not for urination ($F_{(2, 135)} = 2.58$; $p = 0.08$).

As in first occurrence of open field, statistical analysis revealed a significant dose influence for locomotor activity, where high Na-F group displayed more horizontal locomotion (Fig. 2a), ($F_{(1, 135)} = 4.51$; $p = 0.04$) and higher vertical activity (Fig. 2b), ($F_{(1, 135)} = 13.43$; $p = 0.00$) compared to control group. In addition, high Na-F groups were the most anxious group as indicated by duration of freezing (Fig. 2c), ($F_{(1, 135)} = 5.72$; $p = 0.02$), defecation ($F_{(1, 135)} = 9.69$; $p = 0.00$) and urination scores ($F_{(1, 135)} = 5.08$; $p = 0.03$). Again, non of the measured parameters were affected by duration of exposure to Na-F. Hence, over the three test sessions, impairment in habituation was markedly seen in high Na-F group compared to other treatments.

3.3. Maze test

During acquisition on the first day of testing, significant differences among treatments were recorded regarding time elapsed and numbers of entries of blind alleys. Animals received high Na-F doses required more time to locate the feed at the end of the maze ($F_{(1, 45)} = 19.37$; $p = 0.00$) with higher frequency of entering blind alleys ($F_{(1, 45)} = 8.25$; $p = 0.01$), compared to control group. On the other hand, duration of exposure to Na-F had only a significant influence on time spent in maze test ($F_{(1, 45)} = 6.45$; $p = 0.02$).

Over the five days of maze test, all treatments required progressively less time to locate feed ($F_{(4, 225)} = 24.45$; $p = 0.00$). Similar decline trend was also noted for numbers of entries of blind alleys ($F_{(4, 225)} = 34.25$; $p = 0.00$). Learning and memory assessed over five days of maze test showed that groups of animals exposed to high concentrations of Na-F for long period took longer time to locate feed (Fig. 3a), (dose effect, $F_{(1, 225)} = 16.13$; $p = 0.00$), exposure time effect ($F_{(1, 225)} = 15.21$; $p = 0.00$) with higher frequency for entering blind alleys (Fig. 3b), (dose effect, $F_{(1, 225)} = 29.54$; $p = 0.00$), exposure time effect ($F_{(1, 225)} = 26.73$; $p = 0.00$), demonstrating poorer memory retention relative to all other treatments.

3.4. Novelty exploration test

After re-exposure to the novel environment, habituation occurred as evidenced by decreased exploratory activities in all treatments compared to their values during initial exposure. Numbers of both rearing and head dipping were significantly lower during re-exposure ($F_{(1, 90)} = 34.71$; $p = 0.00$) and ($F_{(1, 90)} = 13.40$; $p = 0.00$), respectively.

A significant discrepancy in exploratory activities was found between treatments during both novel exposure and re-exposure. Group of rats administered high Na-F concentrations for long period reared more compared to other groups (Fig. 4a), (dose effect, $F_{(1, 90)} = 17.53$; $p = 0.00$), nevertheless, time of exposure to Na-F had only a marginal significance ($F_{(1, 90)} = 45.12$; $p = 0.05$). The same tendency was also shown for head dips with higher numbers observed in group of animals exposed to high Na-F for long duration (Fig. 4b), (dose effect, $F_{(1, 90)} = 27.09$; $p = 0.00$), exposure time, $F_{(1, 90)} = 6.59$; $p = 0.01$). Thus, a less degree of habituation was noticeably shown in rats with long exposure to high doses of Na-F compared to other treatments.

3.5 Histopathological examination

No pathological changes were detected in the brain of rats in the control group.

The histopathological changes in both treated groups were more or less the same but differ only in their degree of severity. It was obvious and severe in group of rats administered high doses for long duration. The main histopathological changes observed in the brain of rats in both treated groups were congestion of the meningeal, cerebral, cerebellum blood capillaries and choroid plexus (Fig. 5). Areas of hemorrhage in cerebral cortex, cerebellum white matter and in ventricles around choroid plexuses were constant finding in the brain of rats exposed to high Na-F for long duration (Fig. 6). Neurodegenerative changes were detected in nerve cells especially in pyramidal cells of Ammon's horn of hippocampus. The pyramidal cells showed atrophy and necrosis (Fig. 7). Large nerve cells of cerebral cortex showed neurofilaments accumulation in the cytoplasm and the axons. Also, nerve cells of cerebral cortex revealed central chromatolysis, edema, atrophy, necrosis and neuronophagia (Fig. 8), whereas gliosis either focal or diffuse were observed (Fig. 9). Demylination of the nerve fibers in the neuropil was detected in cerebrum accompanied with axonal swelling (Figs. 10 & 11). Signs of encephalitis were observed in the cortex of cerebrum represented by necrotic areas with mononuclear cells aggregations mainly microglial and macrophages cells with perivascular cuffing only in rats exposed to high Na-F for long period (Fig. 12). Glial fibers was detected under the ependymal cells lined the ventricles. The cerebellum showed necrosis of Purkinje cells and edema with necrosis in the granular cell layer in group treated with high dose (Fig. 13). There was an increase in Glial Fibrillary Acidic Protein

(GFAP) expression in astrocytes represented by astrogliosis and astrocytosis in the cerebrum, especially hippocampus. Some astrocytes showed shrinkage of cell bodies and retraction of their processes (Fig. 14).

4. Discussions

Contrary to previous studies that showed no effect of fluoride on body weight (Collins et al., 1995; Chioca et al. 2008; Pereira et al., 2009), lower body weight gain was observed in our study in rats exposed to high daily doses of sodioum fluoride. Similar results derived from other studies with fluoride treated animals (Paul et al., 1998; Ekambaram and Paul, 2001, 2003; Wang et al., 2004). A concomitant reduction in fluid and water consumption was recorded in these studies that might account for body weight reduction (Ross and Daston, 1995). In the study of Das et al. (1994), the atrophic gastritis produced by chronic oral administration of sodium fluoride might be attributed to the decrement in feed intake and consequently lower body weights in rats. Further explanation derived from Shupe et al. (1984) and Ekambaram and Paul (2003) who observed white and chalk-like incisors with broken tips in rats treated with sodium fluoride. These may impair both mastication and swallowing process, resulting in decreased feed intake and body weight.

In the present study, the open field test provides simultaneous measures of both exploratory motor activity (EMA) and anxiety during first occurrence training session. In order to investigate the novel environment, rats were engaged in horizontal as well as vertical activities. Significant enhancement in EMA was noted in high Na-F treated animals, however, the actual increase was related to vertical activity (rearing). This can be interpreted on the basis of increased emotionality in high concentration group. Supportive evidence derived from greater time spent freezing in open field in high Na-F treated group with increased number of faecal boli. The latter was considered the most credible criteria for judging anxious animals. In addition, Davies and Redfern (1973) reported that rearing is not simply a manifestation of exploratory behaviour but might refer to emotionality state of animals. Therefore, it might be concluded that individuals treated with high Na-F were more fearful and highly anxious. However, Chioca et al (2008) reported no impairment in locomotor activity in Na-F treated rats during their first exposure to open field, the impairment in EMA stated by Paul et al (1998) and Ekambaram and Paul (2001, 2003) might be contributed to the high daily doses of Na-F (500 ppm) administered to rats.

Long term habituation to a novel environment is one of the most elementary forms of non-associative learning. In this study, where reduction in spatial exploration during test session was taken as an index for memory habituation (Montag-Sallaz et al., 1999), an impairment in the open field habituation was noticed in Na-F treated group, a result similar to Chioca et al. (2008) using the same concentrations of 50 and 100 ppm Na-F. A proof that learning has taken place is evidenced by habituation on re-exposure to novel environment (File and Wardill, 1975; Platel & Porsolt, 1982). Therefore, sodium fluoride intoxication may impair learning and memory in the present study.

Novelty acquisition during exploration, a hippocampus–dependent phenomenon, has been described as representing a form of information storage (Eichenbaum, 1996; Manahan-Vaughan and Braunewell, 1999). Confirming the data of open field, novelty acquisition test revealed a reduced degree of habituation in rats exposed to high concentrations of Na-F for long duration. Similar results reported by Bera et al. (2007), where impairment of learning was evidenced by reduced habituation on re-exposure to a novel environment in Na-F intoxicated rats.

Concerning the associative learning, memories are based on the acquisition of a predictive link between a specific event and a stimulus. Corresponding to the results of Bhatnagar et al. (2002), where fluoride intoxicated rats performed poorly in maze test, rats with long exposure to high Na-F in the present study demonstrated higher latency with increased numbers of errors in the maze reflecting a poorer memory retention relative to other treatments.

In rats, the hippocampus is involved in learning and memory (Riedel et al. 1999; Wolfman et al., 1999; Vianna et al., 2000; Pittenger et al., 2002, Vianna et al., 2008). Since fluoride is classified as neurotoxic substance, our histopathological examination of the brain confirmed that hippocampus is the most affected region due to fluoride intoxication. This could be attributed to the accumulation of fluoride in different parts of brain in rats especially in hippocampus (Burgstahler and Colquhoun, 1996, Varner et al., 1998). Where fluoride is a chemically active ionized element, it may affect oxygen metabolism and induce oxygen free radicals which appears to play a role in diminishing cognitive ability processes such as learning and memeory (Chirumari and Reddy, 2007). Moreover, fluoride binds antioxidants in the body such as N-acetyl cysteine (NAC) and glutathione (GSH)) and other free-radical destroying enzymes, triggering oxidative stress that leads to cell damage and even cell apoptosis (Rzeuski et al., 1998 and Anuradha et al., 2000). Absence of the compensatory antioxidant system with the presence of oxidative stress due to increased free radicals plays a great role in initiation of damage of nerve cells membrane especially via increased lipid peroxidation (Guan et al., 1998, Ghiselli et al., 2000, and Gao et al., 2009).

The presence of neurofilament in nerve cells as well as the detected increase in expression of Glial fibrillary Acidic Protein (GFAP) in astrocytes may be as a result of interference of fluoride in various steps of protein synthesis inside nerve cells (Miu et al., 2003). Enhanced expression of GFAP has been evidenced to be associated with gliosis and genetic response of CNS to neural injury (Roberts et al., 1989). In addition, Phyllis et al. (1995) has accounted that fluoride may accumulate in both neurons and astrocytes resulting in strong morphological changes, clustering, degeneration and finally death. In addition, learning and memory has been associated with hippocampal activity and cholinergic neurotransmission (Izquierdo et al., 1992; Thiel et al., 1998; Leussis and Bolivar, 2006). Thiel et al. (1999) related the increased release of acetylcholine in the hippocampus to Na-F intoxication in rats, where fluoride may get through the blood brain barrier and accumulate in rat hippocampus resulting in inhibition of cholinesterase activity. Nicotinic acetylcholine receptors (nAChRs) have been also established to play a major role in cognitive processes such as learning and memory. In a fluoride toxicity study for Long et al. (2002), a decreased number of nAChRs was noticed resulting in brain dysfunction. All previous justifications might be responsible for the suppressive effect induced by Na-F intake on learning abilities monitored in the present study.

In conclusion, our study indicated that long exposure for high concentrations of Na-F resulted in clear deleterious effects on brain of rats as reflected in impaired learning and memory.

Since, maternal fluoride ingestion constitutes a great threat to progeny, caution should be exercised when products containing fluoride are administered to nursing mothers.

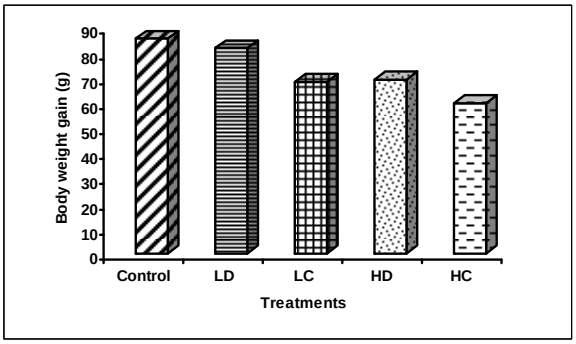
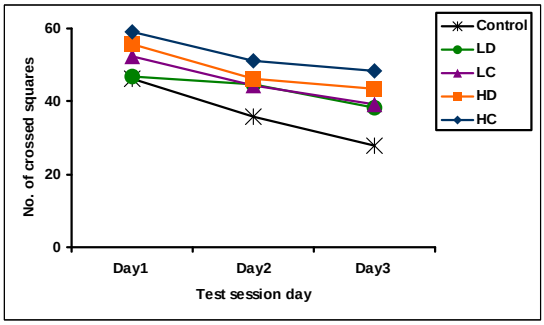
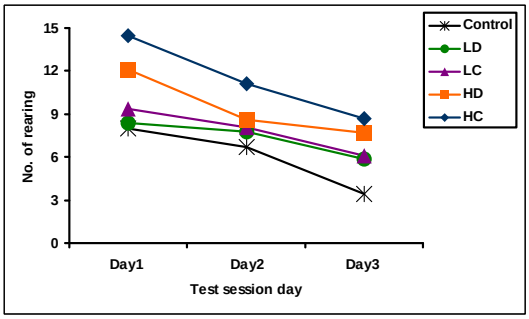


Figure 1: Effects of exposure to different doses and durations of Na-F on body weight gain of rats. Data are presented as mean of 10 animals per treatment.

a) Horizontal activity:



b) Vertical activity:



c) Freezing time:

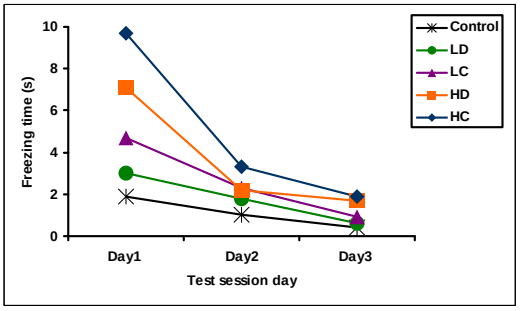
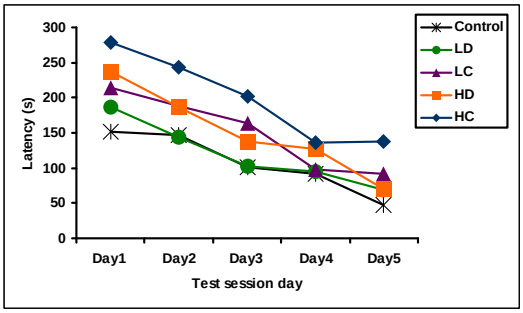


Figure 2: Effects of exposure to different doses and durations of Na-F on measurements of open field test. Data are presented as mean of 10 animals per treatment.

a) Latencies:



b) Numbers of entries of blind alleys:

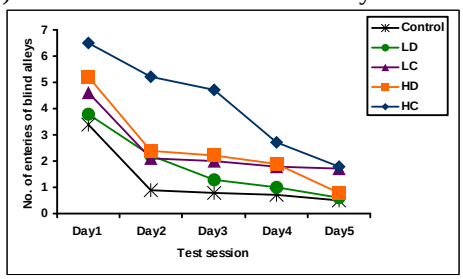
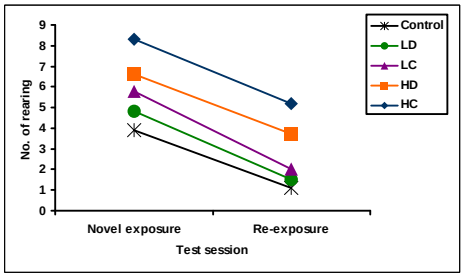


Figure 3: Effects of exposure to different doses and durations of Na-F on measurements of maze test over the course of five days in rats. Data are presented as mean of 10 animals per treatment.

a) Numbers of rearing:



b) Numbers of head dipping:

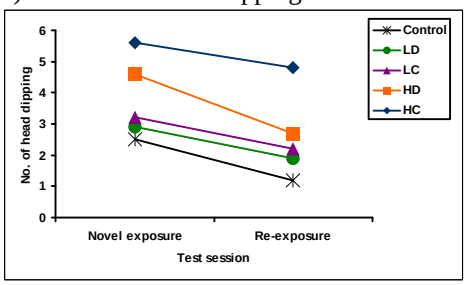


Figure 4: Effects of exposure to different doses and durations of Na-F on measurements of novelty acquisition test in rats. Data are presented as mean of 10 animals per treatment.

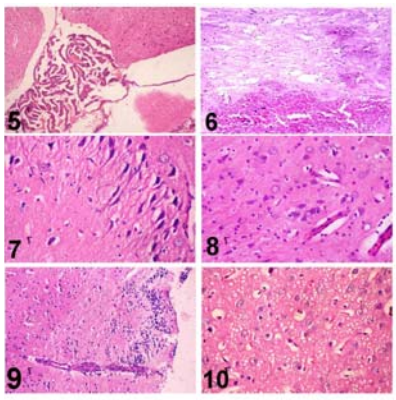


Figure 5: Brain of rat administered high dose of Na-F for long duration showing congestion of choroid plexus, H&E X 100.

Figure 6: Brain of rat administered high dose of Na-F for long duration showing hemorrhagic area in cerebrum, H&E X 100.

Figure 7: Hippocampus of rat administered high dose of Na-F for long duration showing atrophy and necrosis of pyramidal cells H&E X 100.

Figure 8: Brain of rat administered high dose of Na-F for long duration showing necrosis of nerve cells with neuronophagia, H&E X 400.

Figure 9: Brain of rat administered low dose of Na-F for short duration showing focal gliosis, H&E X 100.

Figure 10: Brain of rat administered high dose of Na-F for long duration showing demylination. Notice the cellular edema in the nerve cells, H&E X 400.

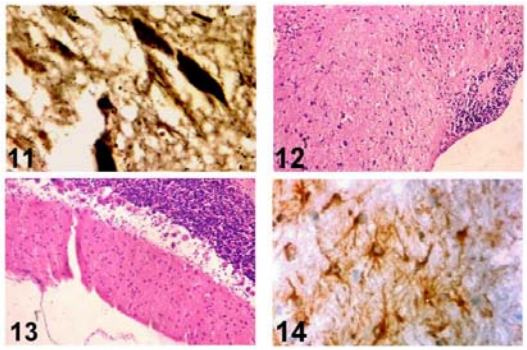


Figure 11: Brain of rat administered high dose of Na-F for long duration showing axonal swelling, Bielschowsky's stain X 400.

Figure 12: Brain of rat administered high dose of Na-F for long duration showing signs of encephalitis, H&E X 100.

Figure 13: Brain of rat administered high dose of Na-F for long duration showing necrosis of Purkinje cells of cerebellum, H&E X 200.

Figure 14: Hippocampus of rat administered with high dose of fluoride showing astrogliosis and astrocytosis stained with Anti-GFAP using DAB chromogen X 400.

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2/1/2010